Reduced effect of pH on skinned rabbit psoas muscle mechanics at high temperatures: implications for fatigue

Edward Pate, Meenesh Bhimani*, Kathy Franks-Skiba* and Roger Cooke*

*Department of Pure and Applied Mathematics, Washington State University, Pullman, WA 99164 and *Department of Biochemistry and Biophysics, and CVRI, University of California, San Francisco, CA 94143, USA*

1. Inhibition of actomyosin function by decreased pH has been proposed to account for much of the depression of muscle function during fatigue. The clearest support for this hypothesis has been from studies of skinned skeletal muscle fibre mechanics at low temperatures ($\leqslant$15 °C).

2. We re-examined the effect of decreased pH (7·0–6·2) on skinned mammalian skeletal fibre mechanics at low (10 °C) and high (30 °C) temperatures, using recently developed protocols that allow reproducible mechanical data to be obtained at higher temperatures.

3. At 10 °C we duplicated previous observations of a significant inhibition of maximum shortening velocity (V_{max}) and isometric tension (P_o) by acidosis. In contrast, at the higher temperature, we found only a very minimal effect of acidosis on V_{max} and a threefold reduction in the decrease in P_o.

4. Thus at temperatures only slightly below physiological for mammalian skeletal muscle systems, pH plays a much less important role in the process of muscle fatigue at the cross-bridge level than has been suggested by data obtained at physiologically unrealistic temperatures.

Following sustained use, muscle enters a state of fatigue characterized by inhibition of peak tetanic tension, twitch tension and the maximum shortening velocity (for recent review, see Fitts, 1994). Despite many years of intense research, the fundamental causes of the diminished mechanical performance of fatigued muscle are not fully understood. Considerable effort has been expended in attempting to correlate changes in the biochemical constituents of the intracellular milieu with downregulation of the myosin motor during fatigue. In particular, intramuscular pH changes from a value of approximately 7 at rest to a value approaching 6 during severe fatigue. This almost tenfold increase in $[H^+]$ has led to suggestions that acidosis may play a significant role in the decreased contractile performance of fatigued muscle. It is this issue that we address here.

Tests of the above hypothesis using living mammalian muscle preparations have been equivocal, with little effect of pH on tension observed at physiological temperatures (Adams, Fisher & Myer, 1991). An inherent limitation of studies in living fibres is that pH may simultaneously affect multiple intracellular functions, making cause and effect relationships between pH and muscle mechanics difficult to sort out. To circumvent this limitation, investigators have employed a demembranated muscle preparation. Here the solution bathing the contractile machinery can then be precisely controlled, overcoming the previously mentioned limitation of living fibre preparations. Using demembranated preparations, we (Cooke, Franks, Luciani & Pate, 1988; Pate & Cooke, 1989) and others (Donaldson, Hermansen & Bolles, 1978; Metzger & Moss, 1987; Chase & Kushmerick, 1988; Kentish & Palmer, 1988; Seow & Ford, 1993) have compared the mechanics of skinned mammalian skeletal fibres contracting under conditions that mimic the pH changes observed during fatigue. The results from different laboratories have been remarkably consistent, with acidosis causing a decrease in both V_{max} and P_o of magnitude comparable to that observed during severe muscle fatigue *in vivo*. Due to the nature of the experimental preparation, this has been widely viewed as the clearest, strongest evidence supporting acidosis as a principal cause of muscle fatigue *in vivo*, acting via a downregulation of the contractile proteins themselves.

A significant limitation of skinned, mammalian skeletal fibre preparations, however, is that they are increasingly unstable mechanically as the experimental temperature is increased, with the most reliable data obtained at temperatures ($\leqslant$15 °C) that are well below the normal physiological range for mammalian muscle. Recently, new 'temperature-jump' protocols have been developed that allow reproducible mechanical measurements to be made on skinned fibres at temperatures more closely approximating

physiological, namely 30 °C (Pate, Wilson, Bhimani & Cooke, 1994). Using these protocols, we have re-examined the effect of pH changes that mimic those observed during fatigue using mammalian fibres, but at both high and low temperatures. Surprisingly, at the higher temperature we found little effect of acidosis on skinned fibre shortening velocity, and a significantly reduced effect on isometric tension. This result helps to reconcile the apparent descrepancy between studies of skinned fibres, which led to the conclusion that pH was a major modifier of cross-bridge function, and studies of living fibres which had led to the conclusion that it was not.

METHODS

New Zealand White rabbits were injected with ketamine hydrochloride (80 mg (kg body weight)$^{-1}$). Following loss of consciousness, the animal was decapitated and exsanguinated. The psoas muscle was harvested and chemically skinned as described previously (Cooke *et al.* 1988). Single fibres were mounted between a solid state force transducer and arm connected to a rapid motor, activated, and mechanical measurements obtained using the 'temperature-jump' protocols described previously (Pate *et al.* 1994). With these protocols, fibres were initially activated at a very low temperature (< 2 °C), at which they generated very low forces and were exceptionally stable mechanically. Following diffusive equilibration of the various buffer species across a fibre at the low temperature, the fibre was rapidly transferred to an equivalent buffer maintained at the higher temperature at which force and velocity data were acquired (10 or 30 °C). Temperature equilibration across a 75 μm diameter fibre is several orders of magnitude more rapid than the traditionally employed diffusive equilibration of Ca^{2+} during skinned fibre activation. Following the temperature jump, maximum force generation requires only 100 ms, a value comparable to that observed with mammalian fibres *in vivo*. We have found the temperature-jump method to provide for more uniform fibre activation at the higher temperature and isometric tension. This has allowed reproducible mechanical data to be obtained at both 10 and 30 °C.

The compositions of the experimental buffers are described in Table 1. Final concentrations were determined from standard binding constants using a computer program described previously (Pate, Nakamaye, Franks-Skiba, Yount & Cooke, 1991). All experiments were done at 5 mM calculated substrate concentration, 200 mM ionic strength, pH 6·2 or 7·0. Skinned fibre P_o is dependent upon $[P_i]$, with greater sensitivity to a given small change in $[P_i]$ at lower $[P_i]$. Contaminating P_i is present in commercially available reagents. Thus, as observed by Millar & Homsher (1990), a moderate $[P_i]$ offers a better, more reproducible standard for mechanics than the more commonly employed solutions with no added P_i. Hence, 5 mM P_i was added to all buffers. P_i concentrations of this magnitude have been observed previously for resting mammalian muscle (Cady, Jones, Lynn & Newham, 1989; Adams, Fisher & Meyer, 1991).

Table 1. Composition of solutions (mM)

	30 °C pH 7·0	30 °C pH 6·2	10 °C pH 7·0	10 °C pH 6·2
Final $MgATP^{2-}$	5·0	5·0	5·0	5·0
Total Na_2ATP	5·5	5·9	5·8	6·3
Final Mg^{2+}	2·3	2·4	2·4	2·4
Total $Mg(OAc)_2$	10	10	10	10
Final CP^{2+}	18	18	18	18
Final $H_2PO_4^- + HPO_4^{2-}$	3·7	4·4	4·2	4·6
Final $K^+ + Na^+$	167	167	162	166
Final OAc^-	86	83	88	86

All activating solutions also contained (Total) (mM): 1·0 EGTA, 20·0 Na_2CP, 40·0 H^+ buffer (Mops at pH 7·0, Mes at pH 6·2), 5·0 KH_2PO_4, 1 $CaCl_2$ (final calculated pCa ≈ 4·5–4·7), 2·0 mg ml^{-1} creatine phosphokinase and variable amounts of potassium acetate to yield a final ionic strength of 200 mM for all activating solutions. pH was adjusted by addition of KOH. For all experiments involving active fibres, small additional aliquots of $CaCl_2$ were added to the experimental buffers at the time of experimentation and fibre tension monitored in order to insure that full activation had been obtained. The computer program additionally determined concentrations of the following additional species in determining the components above and in determining ionic strength: $CaEGTA^{2-}$, $CaATP^{2-}$, Ca^{2+}, ATP^{4-}, $EGTA^{4-}$, MgCP, $MgHPO_4$, $MgH_2PO_4^+$, $CaHPO_4$, $CaH_2PO_4^+$, $KATP^{3-}$, $MgEGTA^{2-}$, $HATP^{3-}$, $MgHATP^-$, $HEGTA^{3-}$, H_2EGTA^{2-}, H_3EGTA^-, H_4EGTA, $CaHEGTA^-$, $MgHEGTA^-$, H_2ATP^{2-}, $CaHATP^-$, $CaPO_4^-$, $MgHPO_4$, $KHPO_4^-$, HCP^-, H_2CP, H_3CP^+, $CaOAc^+$, $MgOAc^+$, HOAc, PO_4^{3-}, $Mops^-$, HMops, Mes^-, HMes. For determining ionic strength, the protonated, zwitterionic pH buffers were assumed to have zero charge (Rees & Stephenson, 1987). We note that [MgCP] ≈ 2 mM for all conditions. The remainder of the phosphate not in the mono- or diprotonated forms was primarily in the $KHPO_4^-$ and $MgHPO_4$ species. Phosphate contamination in commercially available ATP and CP (both from Sigma) resulted in ~0·5–1 mM additional added phosphate (Dantzig, Goldman, Millar, Lacktis & Homsher, 1992). This addional P_i source was not included in determination of final $H_2PO_4^- + HPO_4^{2-}$ or of ionic strength. Abbreviations: CP, phosphocreatine; OAc, acetate.

Fibre length was determined by measurement at ×12 magnification using the graticule of a dissecting microscope. Diameters were measured at ×100 magnification at five to seven locations along the fibre and the values averaged. Isometric tensions were normalized with respect to fibre area. For isotonic releases, shortening velocity was normalized with respect to fibre length. Isotonic releases were done in sets of three, spaced 2 s apart. In each set after the first, one release tension duplicated a release tension from the first set. A change in shortening velocity of >10% in the duplicated release resulted in termination of the experiment. A new fibre was then mounted and activated. Subject to the repetition constraint, an effort was made to otherwise randomize the magnitudes of the other release tensions. Accumulated force–velocity data were fit to the Hill equation (Hill, 1938):

$$V = V_{max}(a/P_o)[1 - P/P_o]/[a/P_o + P/P_o].$$

Computations were done on a Packard Bell model PB286 computer running MS-DOS 3.3 using the non-linear least-squares routine NLIN (modified Gauss–Newton option, equal weighting of data points) in the Statistical Analysis Subroutines package (release 6.03, SAS Institute, Research Triangle Park, NC, USA). A grid search for starting values was employed in an attempt to ensure convergence to the global minimum sum of the squared residuals. At 30 °C (10 °C), each Hill fit represented data from eight (6) different fibres. Additional discussion of experimental technique is in Pate *et al.* (1994).

RESULTS

Following activation of a muscle fibre at either 10 or 30 °C, P_o was determined. The fibre was then allowed to shorten at predetermined fractions of P_o (isotonic releases). Typical fibre length (as determined by the position of the motor arm) and force data as a function of time during isotonic releases are shown in Fig. 1 for fibres contracting at 30 °C. Least-squares linear fits were done to the position data over the period 10–40 ms following release, with the slopes taken as shortening velocities. As can be seen in Fig. 1, for similar release tensions, the linear fits to the position data provided similar slopes (velocities) at pH 7·0 and 6·2. Figure 2 shows the accumulated force–velocity data, obtained by fitting the traces between 10 and 40 ms, and the fit to the Hill equation used to determine V_{max}. The mechanical data are summarized in Table 2. At 10 °C, a decrease in pH from 7·0 to 6·2 decreased V_{max} by ~30% and P_o by ~50%. These results are similar to those from previous studies that have led to the conclusion that acidosis is a major agent in fatigue, acting at the level of the actomyosin interaction (Donaldson *et al.* 1978; Metzger & Moss, 1987; Chase & Kushmerick, 1988; Cooke *et al.* 1988; Kentish & Palmer, 1988; Pate & Cooke, 1989; Seow & Ford, 1993). At the elevated temperature of 30 °C, however, the situation was quite different. V_{max} was not depressed by the decease in

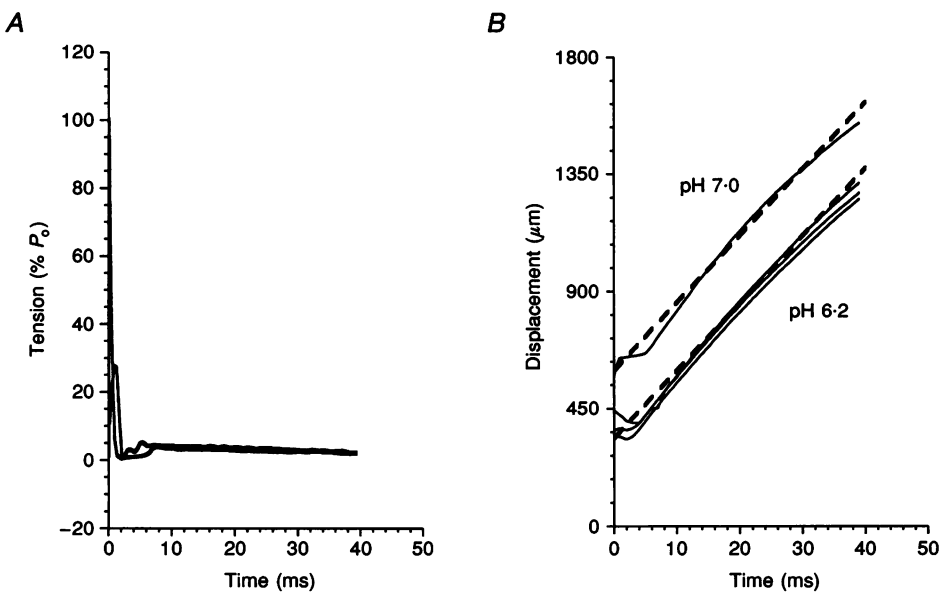

Figure 1. Isotonic release data for chemically skinned rabbit psoas muscle fibres

Fibres were activated at 30 °C and pH 7·0 and 6·2. *A*, release tensions as a function of time following initiation of isotonic releases; *B*, position of the motor arm to which the fibres were attached. The slope of a least-squares fit to the position *vs.* time data over the period 10–40 ms following release is also shown for two of the traces (dashed lines) and was taken as the shortening velocity. At pH 7, the release tension was 2% of P_o, with least-squares slope giving a velocity of 5·9 muscle lengths s^{-1}. At pH 6·2, 3 releases were performed to 4% of P_o for the same fibre with sequential velocities of 5·4, 5·7 and 5·4 muscle lengths s^{-1}, showing data reproducibility at high temperatures using the temperature-jump protocols. The least-squares fit for the fastest of these traces at pH 6·2 is also shown.

Table 2. Mechanical parameters of contraction as a function of pH and temperature

pH	Temp (°C)	P_0 (N mm^{-2})	V_{max} (l s^{-1})
7·0	30	0·28 ± 0·02 (10)	6·28 ± 0·14
6·2	30	0·23 ± 0·02 (7)	6·68 ± 0·23
7·0	10	0·15 ± 0·01 (15)	1·89 ± 0·05
6·2	10	0·07 ± 0·01 (6)	1·33 ± 0·05

Isometric tension and maximum shortening velocity for fibres activated at high and low temperatures for conditions in which pH simulates *in vivo* values for both resting (pH 7·0) and severely fatigued (pH 6·2) mammalian skeletal muscle fibres. Tension is normalized with respect to fibre cross-sectional area. V_{max} (muscle lengths s^{-1}) is determined by extrapolation of the Hill fit of the force–velocity data (Fig. 2) to zero tension. Errors are ± S.E.M. (number of observations) for P_0, and ± asymptotic 95% confidence limits from the Hill fit for V_{max}.

pH; instead it was very slightly elevated (~6%) by our simulated fatigue conditions. At 30 °C, P_0 was still decreased as pH decreased from 7·0 to 6·2. However, the proportional decrease was a much more modest value of only 18%.

Our fit of the data predicts a slight decrease in V_{max} at the higher temperatures. It is not clear to what extent this increase should be viewed as real or potentially a function of the fitting procedure. From Table 2 it is evident that the differences in V_{max} were statistically significant for the Hill-type model we used, which forced the curves to pass through P_0. Other Hill formulations may result in a different change in V_{max}, however. Additionally, the displacement traces as a function of time in Fig. 1 are not linear, but instead display a slight downward concavity. Similar behaviour has been observed for skinned mammalian fibre preparations at lower temperatures. At 30 °C, this curvature is more pronounced at pH 7 than at 6·2. Thus it is important to consider the implications of changing the time period over which the least-squares fit is made to the position data. At the extreme, one can scale the 30 ms long fitting period used at 10 °C (10–40 ms following release) by the approximately fourfold velocity increase between 10 and 30 °C and again compare the slopes. Using this approach on the data in Fig. 1 (30 °C) and fitting the position data between 8 ms (the earliest time following release at which the tension has stablized) and 16 ms, the fibres are contracting only 11% faster at pH 7 than at pH 6·2. Thus the effects of curvature do not change our primary conclusion that pH has little effect on velocity at 30 °C.

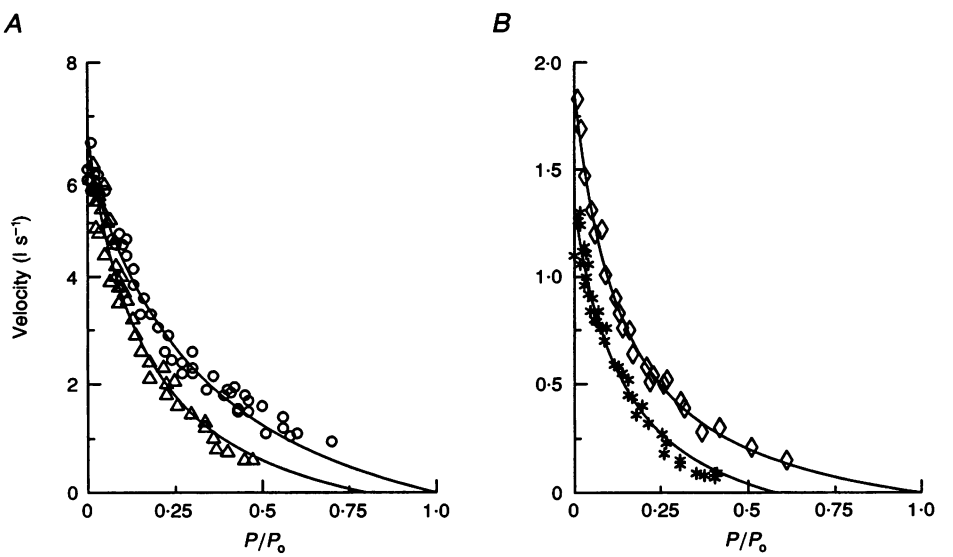

Figure 2. Accumulated force–velocity data

Accumulated force–velocity data for fibres contracting at 30 °C and pH 7·0 (○) and pH 6·2 (△) (*A*); and 10 °C and pH 7·0 (◇) and pH 6·2 (✱) (*B*). The continuous lines are least-squares, hyperbolic Hill fits to the force–velocity data. For each temperature, tensions (horizontal axis) are normalized with respect to the isometric value obtained for fibres contracting at pH 7·0; velocities are in muscle lengths per second. Note the change in vertical scale between *A* and *B*.

In determinations of P_0, we have previously observed that the largest source of error was in the determination of the fibre cross-sectional area. As an additional control on the observed decrease in P_0 with pH at 30 °C, the following paired measurement was employed. Fibres were initially activated at pH 6·2 (or 7·0) using the temperature-jump protocol, and P_0 determined. The fibre was then relaxed (< 2 °C) and after 2 min reactivated at the other pH, allowing it to serve as its own control for cross-sectional area. We obtained a 17·4 ± 2·5% (mean ± S.E.M., four observations for each direction of pH change) decrease in P_0 at pH 6·2 when compared with pH 7·0. This value was virtually identical to that in Table 2, permitting additional confidence in our results.

DISCUSSION

Using chemically skinned rabbit psoas fibres, we have demonstrated a temperature-dependent effect of acidosis (pH 7–6·2) on muscle mechanics. At 10 °C we find that acidosis has a significant inhibitory effect on both P_0 and V_{max}. In contrast, at the closer to physiological temperature of 30 °C, there was a diminished effect of acidosis on P_0, and our least-squares fits for V_{max} indicated that, if anything, V_{max} increased very slightly at the lower pH. Although it may appear unusual that a product of hydrolysis should increase a rate rather than inhibit it, there have been previous observations of such behaviour for another substrate hydrolysis product. At 10 °C and millimolar substrate concentrations, increased $[P_i]$ (>10 mM) increases V_{max} slightly with either MgATP or a non-nucleoside triphosphate as substrate (Pate & Cooke, 1989; Pate *et al.* 1991).

The 53% decrease in P_0 observed at 10 °C is reduced by a factor of three at 30 °C. Our experimental pH of 6·2 is at the lower end of values that have been observed in living mammalian skeletal muscle (Adams, Fisher & Meyer, 1991). Thus our conditions can be regarded as those present during extreme fatigue. Previous observations with both skinned and living mammalian skeletal muscle have indicated that P_0 decreases approximately linearly with pH (Chase & Kushmerick, 1988; Cooke *et al.* 1988; Westerblad & Allen, 1993). Thus less severe acidosis would be expected to reduce our observed 18% decrease to an even smaller value.

Experimental investigation of the influence of H^+ on the actomyosin interaction in solution has been limited. However, there are reasons to expect that pH would have such a temperature-dependent effect. Taylor (1977) has shown that decreased pH shifts the equilibrium of the ATP hydrolysis step by myosin towards the myosin–ATP state. Because a myosin–ADP–P_i state bound to actin is the one that precedes tension generation (Hibberd & Trentham, 1986), such a shift would be expected to decrease tension. The magnitude of the observed equilibrium shift to the myosin–ATP state by acidosis decreased with increasing temperature. Thus at least a portion of the temperature dependence of the effects of pH may be due to a difference in its ability to reverse the hydrolysis step. If a similar equilibrium shift occurs between weakly bound ATP and ADP–P_i states that produce different amounts of resistive drag on sliding velocity, a temperature-dependent effect on V_{max} could also occur. As observed by Kentish (1991), there may be at least three steps in the actomyosin cycle where hydrogen ions are involved. Thus other steps may be involved as well in the temperature-dependent effects of hydrogen ions on mechanics.

The living muscle cell is complex, and it is not our intention to rule out any involvement of acidosis in the process of muscle fatigue. Clearly, however, our results do require a re-evaluation of the previous conclusions drawn from skinned, mammalian fibre studies at low temperatures. These have strongly supported the role of acidosis in muscle fatigue. At the more physiological temperature, V_{max} is only slightly changed and P_0 is decreased by 17–18% by conditions simulating severe acidosis. Thus the data indicate that at the cross-bridge level, a substantial alteration in pH has at best only a very minor influence on the fatigue process, via a slight depression of P_0. Even here, however, a cautionary note is in order. Our highest experimental temperature of 30 °C is still below the 39 °C internal body temperature of a rabbit. An additional increase in temperature may diminish even further the effect of pH on P_0.

As noted previously, studies with living mammalian fibres have yielded ambiguous results with regard to the relationship between acidosis and muscle fatigue. There is a reasonable correlation between maximum force and intracellular pH during a fatiguing contraction at physiological temperature, which has led some investigators to conclude that there is a cause–effect relation. However, in some of these same studies little correlation is observed during recovery, with force recovery occurring significantly faster than pH (Miller, Bosca, Moussavi, Carson & Weiner, 1988; Cady *et al.* 1989). In addition, direct intracellular acidosis induced by lowering the extracellular pH, has very little effect on tension at physiological temperatures (Adams *et al.* 1991), and only a modest effect at 25 °C (Westerblad & Allen, 1993). These results are in apparent conflict with numerous studies of skinned mammalian fibres discussed above, in which pH appears to be a strong inhibitor of maximum force. Westerblad & Allen attributed this apparent conflict to a fundamental difference between living and skeletal fibres. However, the present results, along with the work cited below, suggest that a more likely explanation for the apparent descrepancy between living and skinned fibres is that the studies of skinned fibres were done at lower temperatures. Our present observations of muscle force are consistent with those of Ranatunga (1987), who measured force as a function of temperature using living rat muscle. Here it was found that lowering extracellular pH from 7 to 6·5 depressed isometric tension at low

temperatures (12–18 °C), but potentiated tension at temperatures approaching physiological (30–35 °C). Unfortunately contraction velocity has not been directly measured in living fibres as a function of temperature and pH.

The situation in amphibian muscle may differ from that in mammalian. Acidosis does inhibit force in amphibian muscle at 20 °C, a physiological temperature for these animals (Renaud, Allard & Mainwood, 1986). However, this inhibition is insufficient to explain the total observed decrease in force during fatigue, indicating that other factors must also play a role in these muscles (Renaud *et al.* 1986; Baker, Brandes & Weiner, 1994).

In summary, in mammalian muscle a unifying theme between skinned and living fibre studies may be emerging, at least with respect to maximum force: acidosis does not significantly affect force generation by mammalian muscles at physiological temperatures. Our results suggest that acidosis, similarly, does not affect V_{max}.

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Acknowledgements

We thank A. Baker and S. Lehman for helpful comments on the manuscript. This work was supported by grants HL32145 (R.C.), and AR39643 (E.P.) from the United States Public Health Service, and by a Grant in aid from the Muscular Dystrophy Association (R.C.). E.P. is an American Heart Association Established Investigator.

Received 22 September 1994; accepted 30 January 1995.